ESTABLISHMENT OF AN 'IN VITRO' MODEL OF DENGUE INFECTION FOR THE STUDY OF IMMUNOLOGIC EVENTS IN THIS DISEASE

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Pathologic studies have shown that dengue virus replicates in organs rich in lymphocytic tissues. Studies in vitro with monkey lymphocytes demonstrated that viral replication is dependent upon blast transformation. Therefore, in the past year, the replication of dengue-2 virus in three human lymphoblastoid cell lines of B cell characteristics in continuous culture (Raji, 8866, Wil 2WT) and in human peripheral lymphocytes (HPL) stimulated by mitogens was studied. Dengue-2 virus (SEATO strain) readily replicated in the three human lymphoblastoid cell lines without apparent effect on the host cells. Persistently

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infected cells produced virus for at least 5 months. The kinetics of the infection and the localization of the dengue antigens were studied by immunofluorescence and electron microscopy. Stimulated and non-stimulated HPL infected after 3 days in culture replicated dengue virus. In contrast, non-stimulated HPL inoculated immediately after isolation did not support virus replication. Persistently infected Raji cells expressing viral antigens on their surface formed rosettes with normal human T lymphocytes and bound human platelets in the presence of human antibody to dengue virus. Immune complexes in sera of patients with dengue could be demonstrated and serum enzyme inhibitors were depressed.

Summary

Pathologic studies have shown that dengue virus replicates in organs rich in lymphocytic tissues. Studies in vitro with monkey lymphocytes demonstrated that viral replication is dependent upon blast transformation. Therefore, in the past year, the replication of dengue-2 virus in three human lymphoblastoid cell lines of B cell characteristics in continuous culture (Raji, 8866, Wil 2WT) and in human peripheral lymphocytes (HPL) stimulated by mitogens was studied. Dengue-2 virus (SEATO strain) readily replicated in the three human lymphoblastoid cell lines without apparent effect on the host cells. Persistently infected cells produced virus for at least 5 months. The kinetics of the infection and the localization of the dengue antigens were studied by immunofluorescence and electron microscopy. Stimulated and non-stimulated HPL infected after 3 days in culture replicated dengue virus. In contrast, non stimulated HPL inoculated immediately after isolation did not support virus replication. Persistently infected Raji cells expressing viral antigens on their surface formed rosettes with normal human T lymphocytes and bound human platelets in the presence of human antibody to dengue virus. Immune complexes in sera of patients with dengue could be demonstrated and serum enzyme inhibitors were depressed.

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Introduction

Two distinct syndromes are associated with dengue virus (a group of antigenically related members of the group B arthropod-born virus family) infection: Dengue fever and dengue hemorrhagic fever (DHF). The association of DHF with a second, heterologous dengue virus infection suggested that immunopathologic processes play a major role in this disease (1). Thus, it has been demonstrated that, through an as yet undefined mechanism (possibly immune complexes) the complement system as well as the clotting system is activated in DHF (2). It is conceivable that rapid consumption of complement via the classical and alternate pathway (2,3) results in release of biologically active peptides from C3 and C5 (anaphylatoxins). Massive activation of enzymes of the complement and clotting system may result in relative depletion of serum enzyme inhibitors. It is probable, therefore, that when enzyme inhibitors are depleted via the massive enzyme activation the equilibrium is disturbed and then severe shock occurs.

The above described mechanisms may be triggered by immune complexes free in the circulation or on surfaces of cells. Immune complexes may bind to the lymphocytes and thrombocytes via receptors for IgG Fc, receptors for C3b and C3d and receptors for the virus. Alternatively, virus may enter cells, replicate and be expressed on the cell surface where antibody and complement may be attracted. The final result in both situations will be cell lysis and release of different biologically active mediators as lymphotoxin, MIF, chemotactic factors, and interferon from lymphocytes or serotonin and procoagulant activity from platelets.

A variety of alterations involving lymphocytes takes place during the course of naturally occurring and experimentally produced viral infections. These consist of morphologic changes, altered distribution of lymphocytes and reduction in the cell mediated immune response (4). Lymphoid cells may harbor and release dengue virus. It is known from pathologic studies that dengue virus replicates extensively in organs rich in lymphocytic tissues and that during the course of infection, virus can be isolated from or localized to cells of the monocytic or lymphocytic series (5). Furthermore, Halstead and co-workers have shown

that dengue virus can cause transformation and DNA synthesis in lymphocytes obtained from monkeys after dengue infection (5). Viral replication was shown to be dependent upon changes of lymphocytes accompanying blast transformation. Furthermore, pathologic examination of humans dying of dengue shock syndrome shows a marked lymphocyte necrosis and depletion of the thymus dependent areas of lymph nodes and spleen (6). Also, dengue viral antigen has been found by immunofluorescence on the surface of human mononuclear leukocytes and leukocytes derived from patients with severe dengue infection had significantly more surface Ig than control cells (suggesting the presence of attached immune complexes) (6,7). In this report we shall describe our efforts to study the underlying pathogenetic mechanisms of dengue shock syndrome.

Studies with Human Lymphoblastoid Cell Lines

Human lymphoblastoid cells in continuous culture represent a stimulated cell population and therefore resemble the specifically or nonspecifically (with mitogens) stimulated monkey lymphocytes which were proven to be efficient for supporting dengue virus replication. It was felt, then, that if human lymphoblastoid cells could support dengue virus replication, a model would have been established by which the effect of dengue virus on human lymphocytes as well as the replication of virus, its release and the interplay of viral antigens (released or on cell surface) with antibody and complement could be monitored and a controlled search for mediators of shock could be undertaken. Therefore the following experiments were performed.

a) Absorption of dengue-2 virus by human lymphoblastoid cells. Four human lymphoblastoid cell lines (Raji, Daudi, 8866 and Wil2WT) were incubated at a multiplicity of 0.1 with mouse brain seed of the dengue-2 (New Guinea strain C) for 90 min at 37° C. At the end of the incubation period, cells were separated by centrifugation and an aliquot of the cell pellet (after disrupting the cells by freezing and thawing) as well as of the supernatant was checked for the number of PFU by the plaque assay (8). Similar experiments were performed with the Raji cell line by incubating these cells with dengue-2 virus from mouse brain seed, from $LLCMK_2$ monkey kidney monolayer seed and from Raji-$LLCMK_2$ seed. The results of the above absorption experiments are summarized in Table I.

Table I

Absorption of Dengue-2 virus
by human lymphoblastoid cell lines

Cell line	Source of Virus		
	Mouse brain seed	$LLCMK_2$ seed	Raji-$LLCMK_2$ seed
	%	%	%
Raji	41	29	75
Wil2WT	21	ND	ND
8866	24	ND	ND
Daudi	74	ND	ND

15×10^6 cells from each cell line were incubated at 37°C, 90 min, with Dengue-2 virus obtained from different sources at a multiplicity of 0.1. From the input PFU (corrected for inactivation of virus by incubation at 37°C for 90 min) the percent of virus absorbed on the cell pellets was determined.

It can be seen that all four cell lines absorbed dengue virus from the mouse brain seed and that the absorption of Raji-LLCMK$_2$ virus (SEATO strain) on Raji cells was higher than that from the other viral sources. The results indicated that all three cell lines have receptors for dengue-2 virus.

b) Replication of dengue-2 virus in human lymphoblastoid cell lines. After establishing that Raji-LLCMK$_2$ dengue-2 virus is absorbed on the surface of human lymphoblastoid cells, the replication of the virus in these cells was followed. Cells were infected with dengue virus at a multiplicity of 0.1 and then divided in aliquots and cultured in medium. At 0 time and 24 hour intervals, supernatant and cell pellet was checked for the number of PFU by the plaque assay. As is shown in Table II, virus replicated in all three cell lines.

Table II

Replication of Dengue-2 virus (Raji-LLCMK$_2$) in human lymphoblastoid cell lines

Time (hours) post inoculation	PFU/10 ml culture					
	Raji-Sup	Raji-Cells	Wil2WT-Sup	Wil2WT-Cells	8866 Sup	8866-Cells
0	2.5×10^2	1.3×10^2	0	3.3×10^2	0	6.5×10^2
24	0	1.0×10^1	1.0×10^3	1.6×10^3	1.4×10^3	2.8×10^3
48	14×10^2	1.1×10^3	4.0×10^2	9.6×10^2	8.5×10^2	1.6×10^4
72	8.8×10^3	7.6×10^3	2.3×10^4	8.6×10^3	9.5×10^2	5.2×10^4
96	9.2×10^4	5.7×10^4	1.3×10^4	3.2×10^5	8.5×10^2	1.9×10^4
120	9.8×10^4	8.8×10^4	9.9×10^3	2.5×10^5	5.5×10^2	6.6×10^3
144	1.0×10^5	1.2×10^5	-	-	4.0×10^2	2.8×10^3

The viral titers intracellularly and extracellularly were similar for the Raji and Wil2WT cell lines, whereas with the 8866 cells much less virus was released in the supernatant. The results indicated that dengue virus replicates in human lymphoblastoid cells and it is released in the medium.

c) Kinetics of the infection. The kinetics of the infection was studied by an indirect immunofluorescence technique. Mouse hyperimmune ascitic fluid containing antibodies to dengue-2 virus was used. Cells were incubated with the above antiserum (ascitic fluid) or normal mouse ascitic fluid and then reacted with FITC rabbit antimouse gamma globulin. It was found that when 15×10^6 cells were incubated with 3×10^5 PFU of Raji-LLCMK$_2$ virus for 90 min at

37°C, nearly 100% of the cells were membrane fluorescence positive. No staining was observed with noninfected cells or infected cells incubated with normal mouse ascitic fluid. At 24 hours infected cells did not show surface staining. At 48-72 hours, cell surface staining appeared again but was very weak. Cells were negative for cytoplasmic staining at 1-1/2 hours but became weakly positive at 24 hours and well positive at 72-96 hours. The positive surface staining on lymphoid cells incubated with virus for only 1-1/2 hours suggests that an early cytolysis of human lymphocytes might take place in the circulation. Such an early cytolysis has been described for paramyxovirus infected cells (9).

d) Establishment of chronically infected cell lines. Since we observed that lymphoblastoid cell lines support dengue-2 viral replication without any apparent cytopathic effect we attempted to establish chronically infected cell lines. The following experiment was performed. Raji, Wil2WT and 8866 cells were infected with Raji-LLCMK$_2$ virus and then cultured. After 4 days of incubation, infected cells were subpassed. The subpassed cultures were incubated at 37°C for 3 days, at which time the amount of extracellular and intracellular viral titers were determined, and passage was repeated. In Table III the amount of virus found up to 27th passage is shown.

Table III

Replication of Dengue-2 virus (Raji-LLCMK$_2$ strain) in chronically infected human lymphoblastoid cell lines

Passage No.	PFU/10 ml culture					
	Raji-supe	Raji-cells	Wil2WT-supe	Wil2WT-cells	8866-supe	8866-cells
1st	2.0×10^3	4.0×10^4	6.0×10^3	2.3×10^4	2.3×10^3	3.7×10^4
2nd	6.7×10^3	5×10^5	2.4×10^4	3.1×10^4	5.7×10^3	1.2×10^4
3rd	1.9×10^4	3.7×10^5	1.0×10^4	3.7×10^4	1.6×10^3	3.4×10^3
4th	5.0×10^5	3.7×10^5	1.0×10^5	1.2×10^5	9.9×10^3	2.5×10^4
5th	2.5×10^5	1.8×10^4	1.5×10^3	1.0×10^5	4.5×10^4	6.4×10^4
6th	9.8×10^4	1.9×10^4	4.0×10^3	6.5×10^4	6.9×10^4	4.4×10^4
7th	5.5×10^5	1.96×10^5	5.0×10^3	1.8×10^4	7.0×10^3	7.2×10^3
8th	7.0×10^4	9.6×10^4	1.9×10^4	1.0×10^5	3.5×10^3	2.4×10^4
9th	7.5×10^4	11.2×10^4	4.1×10^5	6.1×10^4	5.0×10^3	1.7×10^4
10th	2.2×10^5	3×10^5	4.5×10^5	2.5×10^5	1.7×10^4	5.5×10^4
20th	3.2×10^5	6.2×10^5	1.3×10^4	2.7×10^4	5.3×10^4	1.3×10^4
27th	2.9×10^5	13.4×10^4	-	-	1.7×10^4	2.3×10^4

It can be seen that there was a fluctuation of the viral titers and that both Raji and Wil2WT cells produced more virus than 8866 cells. Virus production continued for the first 52 passages, although the titers in supernatant gradually declined from approximately 5×10^5 PFU/10 ml culture in early passages to 1×10^2 PFU/10 ml culture by the 50th passage. No studies on the virulence of the virus obtained were conducted.

e) Morphological and immunofluorescence studies with chronically infected cell lines. No cytopathic effect on the infected cells was seen. However, a smaller mitotic index was found in infected Raji cells as compared to uninfected cells. Cells appeared large in size and some of them were multinucleated.

Fluorescence studies on the chronically infected cultures revealed the presence of intense cytoplasmic and membrane fluorescence in more than 70% of Raji cells and Wil2WT cells. The percent of positive fluorescent Raji and Wil2WT cells increased in the later passages, which suggests cell to cell spreading of the infection or selective proliferation of infected cells. However, 8866 cells continue to give only 5-10% fluorescence positive cells, as at the start of the infection. The fact that 100% of 8866 cells were positive for surface antigen after initial incubation for 90 min with dengue virus but only 5-10% of the chronically infected cells showed intracellular and membrane staining, indicates that 8866 cells had receptor sites for the dengue virus but the majority of them could not synthesize viral proteins. Such a phenomenon has been described for Raji cells infected with vesicular stomatitis virus (10).

Electron microscopic studies conducted with chronically infected cells showed in some cells a few particles resembling arboviruses inside cytoplasmic vacuoles.

f) Markers for human B and T cells on the infected cell lines. Lymphoid cells of the immune system can be divided into two functional groups: the thymus derived (T) and the bone marrow derived (B) cells. The role of T and B cells in the immune system has been reviewed (11). Lymphocytes have been shown to be the major cell type involved in the cell mediated immune response, and their importance in the host's defense against certain viral infections is illustrated by the severe infections produced by vaccinia and herpes viruses in patients with congenital thymic deficiency syndromes (12). Therefore, we decided to study the characteristics of the infected cell lines as far as their B or T cell origin is concerned. By the criteria used recently (13,14) it was found that all three infected cell lines can be classified as B type (Table IV).

Table IV

Markers for B and T cells on human lymphoblastoid cell lines

Cell Type	% Cells with MBIg	% Cells Binding IgG Fc	C3	C3b	% Rosette Forming Cells EAC1-3b	EAC1-3d	SRBC
Raji	0	100	100	100	100	95	0
Wil2WT	100	0	0	0	2	0	0
8866	80	0	0	0	5	0	0
Daudi	100	100	100	90	2	42	0

EAC1-3b - EA coated with isolated human C1, C4, C2, C3
EAC1-3d - EA coated with human C1, C4, C2, C3 and then rendered immune adherence negative
SRBC - sheep red blood cell
MBIg - membrane bound immunoglobulin

However, this statement can only be made with some reservations. For instance, Raji cells do not carry surface Ig but form rosettes with EAC' (erythrocyte-antierythrocyte antibody-complement) and bind aggregated human gamma globulin (AHG). In contrast, Wil2WT and 8866 cells carry surface bound Ig but do not form rosettes with EAC' and do not bind AHG. None of the above cell lines form rosettes with sheep erythrocytes (a human T cell characteristic).

Currently we are trying to identify a cell line with T cell characteristics and see if it will support viral replication as the B type cell lines do.

Studies with Human Peripheral Lymphocytes

Since human lymphoblastoid cell lines support viral replication, experiments were conducted in which the ability of non-stimulated and stimulated human peripheral lymphocytes to support dengue virus replication was studied. Human peripheral lymphocytes were isolated from one donor and the following experiment was performed.

a) Aliquot of lymphocytes was stimulated for 3 days with PHA-P (phytohemagglutinin) and then infected with dengue-2 virus (Raji-LLCMK$_2$ strain).

b) Aliquot of lymphocytes was cultured for 3 days without PHA-P and then infected with dengue-2 virus.

c) Aliquot of lymphocytes was infected at 0 time (just after isolation) with dengue-2 virus.

The infected cells were washed and divided in aliquots for 0,24,48,72,96,120,144 hours. Viral titers in supernatant and cell pellet were assessed. The results are summarized in Table V.

Table V

Replication of Dengue-2 virus (Raji-LLCMK$_2$) in
PHA stimulated and unstimulated human peripheral lymphocytes

	PFU/2 ml/2.5 x 10^6 cells					
Time (hours) post inoculation	PHA Stimulated Lymphocytes		Nonstimulated cells injected after 3 day culture		Nonstimulated cells infected at 0 time	
	Supe	Cells	Supe	Cells	Supe	Cells
0	15×10^1	1.0×10^1	1.0×10^1	5	0	0
24	3.0×10^1	3.0×10^1	2.0×10^1	5	0	0
48	9×10^3	2.4×10^2	1.0×10^1	5	0	0
72	7×10^3	3.6×10^3	18.0×10^1	3.0×10^2	0	0
96	7×10^3	6.1×10^3	4.0×10^2	1.2×10^3	0	0
120	6×10^3	5.0×10^3	15.2×10^2	3.7×10^3	0	0
144	14.2×10^3	8.2×10^3	11.8×10^3	2.0×10^4	0	0

Both stimulated and nonstimulated cells cultured for 3 days and infected, replicated dengue virus. Stimulated cells showed an early production and release of virus (48-72 hours) whereas nonstimulated cells but cultured for 3 days prior to inoculation with dengue virus, reached similar titers later (120-144 hours). In contrast, nonstimulated cells inoculated immediately after isolation did not support viral replication. The reason that dengue-2 virus replicated in nonstimulated human peripheral lymphocytes which were cultured for 3 days prior to inoculation are not at the present well understood but similar phenomenon has been described with other viruses (4). It may be that alterations of cell surface properties or nonspecific activation of Epstein-Barr virus genome carrying cells is responsible for the support of dengue-virus replication.

Since human peripheral lymphocytes (HPL) absorb dengue-2 virus and support its replication, it was of interest to see if HPL incubated with persistently infected Raji cells (carrying on their surface dengue viral antigens) would adhere to them. Recently, receptors for Epstein-Barr virus on the surface of human B cells have been described (15). When HPL were incubated with chronically infected Raji cells, approximately 10-15% of the HPL were found to be adherent to the Raji cells, sometimes forming rosettes. Incubation of the HPL with fluorescein conjugated antihuman Ig antibody prior to their incubation with Raji cells showed that adherence was not inhibited and that adherent cells were Ig negative (possibly T cells).

Currently we are conducting experiments in which isolated human T or B cells are infected with dengue-2 virus, in order to assess which cell type is responsible for viral replication and if T and B cell collaboration is necessary.

Studies with Human Platelets

Since at the time of dengue hemorrhagic shock syndrome severe thrombocytopenia is found, we also conducted experiments to see the effect of virus, antibody and complement on platelets. Absorption of viruses on platelets, aggregation of platelets by viral-antiviral antibody complexes (16) and release of serotonin and other platelet constituents have been described for other viruses (17). In three consecutive experiments no absorption of dengue-2 virus on platelets could be demonstrated.

Since platelets could be aggregated by immune complexes in the circulation or on the surfaces of cells carrying fixed immune complexes, the following experiment was conducted. Chronically infected Raji cells were incubated with purified human platelets alone or in the presence of mouse or human anti-dengue antibody and complement. The results obtained were equivocal. In some experiments, but not in others, platelets appeared to adhere to chronically infected Raji cells in the presence of antibody. Experiments in which platelets are reacted with virus, antibody and complement and release of radiolabeled serotonin is measured are currently under way.

Studies for the Demonstration of Immune Complexes in Sera of Patients with DHF

As outlined in the introduction, immune complexes may play an important role in the pathogenesis of dengue shock syndrome. Although several methods have been described for the detection of circulating immune complexes in human sera, no method combines the sensitivity

and reproducibility necessary for the identification of small amounts of circulating complexes. Experiments in rabbits have shown that even 10 µg complexes per ml serum can induce severe vasculitis (18). The failure to detect immune complexes in dengue fever so far reflects the limitations of presently available techniques. A sensitive and simple procedure for the detection of soluble immune complexes in sera from patients with various immunological disorders has been recently developed in our laboratory (19). In this method complement receptors on Raji cells are utilized. This method was recently modified so as to become quantitative. Uptake experiments have shown that Raji cells bind 8 times more aggregated human gamma globulin (AHG) via C3b and C3d receptors than via IgG Fc receptors. When Raji cells are incubated with increasing amounts of soluble AHG in fresh or heated serum and then reacted with ^{125}I labeled antihuman IgG it was found that more *Ab was bound to cells incubated with 10 ng AHG in 25 µl of 1:4 fresh serum than cells incubated with 80 µg AHG in heated serum. The ability of Raji cells to detect AHG in serum was dependent on the amount of *Ab used and the size of aggregates. Presence of excess of complement had little effect in the binding of AHG to C3b and C3d receptors. The capacity of Raji cells' complement receptors to bind more efficiently AHG than IgG Fc receptors was used for the detection and quantitation of soluble immune complexes in sera. Sera are incubated with Raji cells, reacted with excess ^{125}I anti-Ig antibody and the amount of radioactivity bound to cells is determined and referred to a standard curve of *Ab uptake by cells incubated with various amounts of AHG in serum. This technique was used to study sera from patients with dengue fever and the results are shown in Table VI.

Table VI

Raji Cell Radioimmune Assay for Immune Complexes in Patients with Dengue Hemorrhagic Fever

Grade of Disease	No. Cases*	No. Positive Cases	Equivalent AHG
			µg/ml**
Grade I	2	1	50
Grade II	8	5	50
Grade III	6	3	100
Grade IV	4	0	0

* 7 serum samples obtained every 24 hours were tested from each patient.

** Mean value.

It can be seen that immune complexes were detectable in several patients expecially with grade II and III of the disease. However, these results will be better understood if serial bleedings of patients with known viral and antibody titers were available, since immune complexes in serum

of dengue patients are present only for a short period of time (20). In the future more detailed studies for the detection of immune complexes in sera from dengue patients with different grades of the disease will be conducted and answers as far as the relationship between presence of immune complexes - complement activation and shock appearance will be obtained.

Studies on the Levels of Serum Enzyme Inhibitors in Sera of Patients with DHF

Activation of enzymes of the complement, kinin and clotting systems in dengue hemorrhagic shock may result in a depletion of serum enzyme inhibitors. We have, therefore, in collaboration with Dr. Hans J. Müller-Eberhard, quantitated the following serum inhibitors in sera from dengue patients by the radial immunodiffusion method: a_2 macroglobulin, inter a trypsin inhibitor, a_1 anti-trypsin, a_1 anti-chymotrypsin. Of all inhibitors, inter a trypsin inhibitor showed the most pronounced decrease. a_2 macroglobulin was also affected but to a lesser degree. The serum concentration of a_1 anti-trypsin was also slightly lowered, whereas a_1 antichymotrypsin remained normal. There was a correlation between depression of inhibitor serum levels and grade of disease. These findings suggest that the cause of severe shock in dengue hemorrhagic fever is the disturbance of the equilibrium between enzyme activation and serum enzyme inhibitor concentration.

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